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Reproductive activities impair immunocompetence in *Physocyclus dugesi* (Araneae: Pholcidae)

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Abstract. When host organisms mount an immune response, they incur energetic costs. Theory predicts that these costs result in trade-offs between investment in life history traits (such as growth and reproduction) and investment in immune response. Recent empirical work investigating whether immune ability is impaired during sexual activity in invertebrates does not uniformly support this prediction. Here, we use lytic activity to test for trade-offs between immune ability and reproductive events in three experiments with the pholeid spider *Physocyclus dugesi* (Simon 1893). First, we test whether males or females have their immune response negatively affected after mating; second, we assess whether oviposition behavior affects immune response; and third, we investigate whether sexual aggression by females affects immune response. We compare reproductive and non-reproductive spiders' immune response. Our results suggest a down-regulation of immune response following mating, oviposition, and aggression. This supports the notion that immunoeompetence is competing for a resource with sexual activities. We discuss reasons why such costs arise in *P. dugesi.*

Keywords: Immune response, mating, oviposition, aggression, trade-off

Parasites are major selective agents such that hosts have evolved a number of adaptations to counter infection. One such adaptation is the immune system, by which hosts recognize self from non-self and act accordingly to eliminate intruders (reviewed by Schmid-Hempel 2011). The immune response imposes both evolutionary and proximate costs (reviewed by Schulenburg et al. 2009; Schmid-Hempel 2011). Given that other life history traits (e.g., traits involved in growth and reproduction) are also costly, infected hosts must face resource allocation dilemmas (Sheldon & Verhulst 1996; Lawniczak et al. 2006). Infections ought to lead to trade-offs where hosts allocate resources to one life history function at the cost of other functions (Schulenburg et al. 2009). The costs of immune responses have been investigated in invertebrates in a variety of contexts with conflicting results.

Researchers of several studies of invertebrates have detected that immune ability may be reduced during or after sexual activity (reviewed by Lawniczak et al. 2006). For example, immune function becomes impaired during copulation (Siva-Jothy et al. 1998; McKean & Nunney 2001; Rolff & Siva-Jothy 2002; Fedorka et al. 2004), oviposition (Siva-Jothy et al. 1998), and male aggressive behavior (Siva-Jothy et al. 1998; Contreras-Garduño et al. 2006). One proximate explanation for decreases in immune ability is that the juvenile hormone directs resources to sexual activities, thereby impairing immune ability (Rolff & Siva-Jothy 2002).

Other studies, however, show no impairment of immune response by sexual activity in invertebrates. For example, immune ability is enhanced during the time of mating in crickets (Shoemaker et al. 2006) and beetles (Valtonen et al. 2010). Individuals may increase investment in immune ability to counter the increased pathogenic risks of mating, such as sexually transmitted diseases (Knell & Webberley 2004). Recent work in damselflies found no down-regulation of immune ability during mating and oviposition, presumably because changes in juvenile hormone concentrations were not sufficient to induce resource allocations that impaired immune ability (Córdoba-Aguilar et al. 2011). Whatever the explanation, these counterexamples indicate that the assumed tradeoff between immunity and sexual function is far from a generalized pattern.

Here, we investigate whether a trade-off exists between immunocompetence and sexual behavior in the round-bodied daddy long leg spider, *Physocyclus dugesi* (Simon 1893). Using three independent experiments, we tested whether mating behavior, oviposition, and agonistic interactions between females affected immunocompetence. As a measurement of immunocompetence, we used lytic activity (LA), a key immune ability variable that has been previously used in studies of ecological immunity in spiders (e.g., Ahtianen et al. 2004, 2005, 2006). Measures of LA quantify the digestive action of a wide variety of antimicrobial peptides activated upon infection. LA is specific in action (Bulet 1999; Genta et al. 2003; Wang et al. 2011), and isoforms of many antimicrobial peptides are present as precursors, allowing rapid immune responses following pathogenic invasions (Hetru 1998). The production of antimicrobial peptides is energetically costly (Ahtianen et al. 2005). We predicted that individuals that mate, oviposit, or interact agonistically during sexual encounters will show reduced levels of LA compared to control individuals.

METHODS

Study species.—*P. dugesi* builds irregular webs that support solitary or group living arrangements of males and females of diverse ages and sizes. Reproductive activity occurs all year

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long, peaking from May to September (Rodríguez-Márquez & Peretti 2010). Mating has a courtship stage that lasts a few minutes, during which both sexes make contact with each other using their legs with vibrating movements along the entire body (Rodríguez-Márquez & Peretti 2010). This is followed by copulation. The entire mating sequence lasts approximately 60 min (Rodríguez-Márquez & Peretti 2010). Fifteen to 20 d following mating, the female lays 10–50 eggs in an egg sac and then holds the egg sac in her chelicerae until the eggs hatch. During this period, females feed infrequently, as otherwise they would have to abandon the egg sac temporarily, which is rarely observed (A. Peretti pers. obs.). Furthermore, egg-sac laden females are aggressive toward other conspecific females that approach (A. Peretti pers. obs.)

Collecting and rearing.—We collected females in their penultimate instar (n = 98) and adult males (n = 38) at the Universidad Nacional Autónoma de México, campus Ciudad Universitaria (19°20′01″N, 99°11′54″W), between April and July 2010. We placed each individual in a plastic container (8 × 12 cm) containing paper (providing a surface for web building) with a wet cotton ball (providing a water source) placed at the base of the container (which was cleaned daily) and maintained these containers at ambient conditions (photoperiod approximately 12/12 h, 11.9–25.5 °C, 57% relative humidity). To collect known virgin females, we maintained juveniles until they reached adulthood. Since experiments were done concurrently, we randomly assigned males and females to specific experiments and treatments. As a measure of body size, we used the tibia-patella length (in mm) of the first pair of legs (cf. Jakob 1994; Huber 1996). For food, we used *Drosophila melanogaster* adults and *Tenebrio monitor* larvae, provided ad libitum once every week. Voucher specimens were deposited in the insect collection of the Instituto de Ecología, Universidad Nacional Autónoma de México.

Estimation of LA.—One of the best ways to measure immunocompetence in arthropods is the assessment of LA (Ellis 1990; Rantala & Kortet 2003; Ahtianen et al. 2004). We drew a sample of 3 ul of haemolymph from each individual by severing its first pair of legs and collecting the haemolymph with a sterile capillary tube. Subsequently, all individuals were stored in 70% ethanol for further measures of body size. Following the technique used by Ahtianen et al. (2004, 2005), each haemolymph sample was mixed with 20 ul buffer (PBS, 0.067 M phosphate, 0.9% NaCl, pH 6.4) and frozen at −80°C. After thawing samples, we pipetted them into an ELISA plate. PBS buffer was used as a negative control. Samples and controls were mixed with 80 ul of a suspension that contained 0.0002 mg/ml of bacteria (liophilized *Micrococcus*, Sigma). We then measured optical density at 492 nm at room temperature in intervals of 1 min. LA was expressed as changes in optical density of a sample after an interval of 10 min; the higher the optical density reading the lower the LA.

Experiments.—*Mating and LA:* We had two groups, experimental (mated animals) and control (unmated animals), to which individuals were assigned randomly. For each mating trial, a virgin female that had just reached adulthood was placed in a larger plastic container (10 × 15 cm, thus facilitating the observer's detection of mating) 24 h before the male, so that the female was able to build the web on which they would copulate. Then we introduced the male. After the male was placed in the container with the female, mating typically commenced after approximately 20 min. There were instances during which neither the male (6 out of 10 cases) nor the female (4 out of 8 trials) were interested in mating. When individuals were unresponsive for 20 min, the disinterested male was replaced by a new individual. All individuals exposed to a mating trial were removed from the experiments, regardless of whether or not they actually engaged in reproductive behavior. Unmated individuals (10 females and 10 males) were treated as mated animals but were never allowed to mate. Females were also introduced to the plastic container indicated above, but no male was introduced, and males were not exposed to any female. Both mated and unmated males were used within 15 d after their capture to reduce effects of potential differences of recent mating histories [for example, a recent mating may affect an individual's immune state, which can be recovered after a few days: for a review in arthropods, see Lawniczak et al. (2006)]. There was no difference in the median sizes of individuals assigned to each treatment (mated males, median 12.709 mm, range 12.132–13.509, vs. unmated males, median 13.24 mm, range 11.711–15.213, Mann Whitney test = −1.109, P = 0.866; mated females, median 10.86 mm, range 10.149–11.982, vs. unmated females, median 11.594 mm, range 10.733–12.147, Mann Whitney test = 0.139, P = 0.448). Each mating lasted exactly 60 min, after which both male and female were removed for immediate haemolymph extraction and LA determination (see above). LA was compared between males that had mated (n = 4) and males that had not mated (control males, n = 10), and between females that had mated (n = 4) and females that had not mated (control females, n = 10).

Oviposition and LA: We selected 10 females collected as adults and placed them in containers (10 × 15 cm). As indicated above, oviposition in these species occurs after approximately 15 d. The day after oviposition we removed the egg sacs and took haemolymph samples from each female. We tested for an effect of oviposition by comparing LA of these females to a control group (n = 10) of known virgin females (collected in their penultimate instars and kept until mature, which was 5 d after they reached adulthood. Size did not differ between these groups (females that oviposited, median 10.967, range 10.119–12.493; females that did not oviposit, median 11.594, range 10.733–12.147; Mann Whitney test = −0.267, P = 0.605).

Agonistic interactions and LA: Agonistic interactions were staged between virgin adult females, which we kept in similar conditions prior to the experiment. One day after reaching adulthood each unmated adult female was placed in a plastic container of 10 × 15 cm for 5 d. Then, one spider (intruder) was removed from its container and placed inside a container that already had a female (resident). We assigned intruder or resident roles at random. We then observed animals continuously for 2 h. Aggression was defined as contact made with any of the legs of both spiders. Aggressive behavior was shown by both spiders in all trials (n = 10). Immediately after each trial, we extracted haemolymph from both females and compared LA of residents (n = 10), intruders (n = 10), and a control group whose females were never exposed to any interaction (n = 10). Residents (median 10.793, range 9.952–

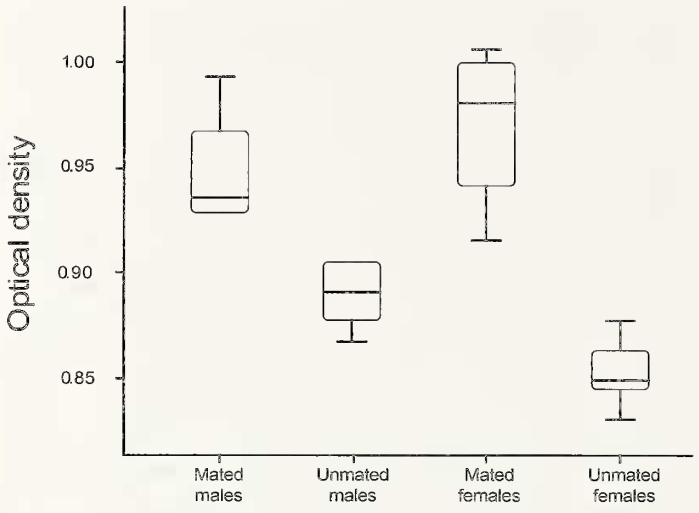

Figure 1.—Optical density readings (of bacterial suspension) according to mating status for both sexes. Each box plot indicates the median ± one quartile; the whiskers show the data range.

11.123), intruders (median 11.227, range 10.19–13.007), and control females (median 11.594, range 10.733–12.147) did not differ in body size (Kruskal Wallis = 2.182, $P = 0.336$).

Statistical analyses.—Due to the small sample sizes in each group, we used Mann Whitney tests to test for LA differences among treatment groups in each experiment. All immune values are given as optical density. We used NCSS 2007 for statistical analysis.

RESULTS

Mating and LA.—Mating was associated with lower lytic activity. Mated males (median 0.935, range 0.929–0.980) showed higher levels of optical density than non-mated males (median 0.891; range 0.875–0.905, Mann Whitney test = 2.466, $P = 0.006$), thus indicating that mated males had lower lytic activity. Similarly, mated females (median 0.980, range 0.929–1.003) showed higher levels in optical density than non-mated females (median 0.849, range 0.840–0.868, Mann Whitney test = 2.557, $P = 0.005$; Fig. 1).

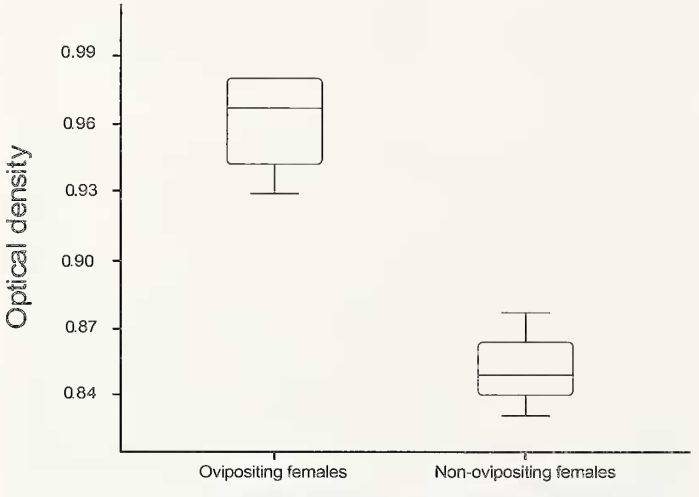

Figure 2.—Optical density readings (of bacterial suspension) in females according to whether they oviposited or not. Each box plot indicates the median ± one quartile; the whiskers show the data range.

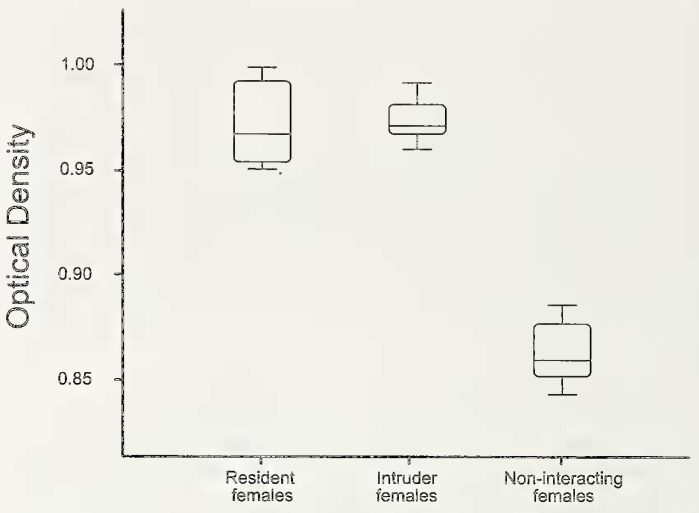

Figure 3.—Optical density readings (of bacterial suspension) in resident, intruder and non-interacting females. Each box plot indicates the median ± one quartile; the whiskers show the data range.

Oviposition and LA.—Oviposition was associated with lower lytic activity. Females that oviposited (median 0.967, range 0.935–0.981) showed higher levels of optical density than females that did not oviposit (median 0.849, range 0.840–0.868, Mann Whitney test = 3.298, $P = 0.0005$: Fig. 2).

Agonistic interactions and LA.—There was no difference in optical density of LA activity between resident (median 0.953, range 0.943–1.037) and intruder females (median 0.962, range 0.957–0.981, Mann Whitney test = 3.738, $P = 0.354$). Given this, we pooled both resident and intruder females and compared them against control females to see whether interacting females (resident and intruder) showed lower levels of LA than non-interacting females (control). Interacting females (median 0.962, range 0.952–0.988) had higher levels of optical density than control females (median 0.849, range 0.840–0.868, Mann Whitney test = 3.722, $P < 0.001$) (Fig. 3).

DISCUSSION

The results of empirical studies are mixed with regard to whether sexual activities such as mating, oviposition, and aggression impair immune ability in invertebrates (reviewed by Córdoba-Aguilar et al. 2011). Despite our small sample size, our results support the hypothesis of a trade-off between immune ability and reproduction and between immune ability and aggressive interactions. Below, we examine the potential causes behind this relationship in terms of resource allocation between immune and sexual functions and the down-regulation of immunocompetence.

Mating reduces immunocompetence in *Physocyclus dugesi*, but such trade-offs may have different explanations in each sex. In males, the energetic cost originates from the behavior males perform during mating, such as movements of the pedipalps, vibrations of the abdomen and leg vibrations (Huber & Eberhard 1997) and intense twisting and squeezing movements using both pedipalps within the female's genital opening (Rodríguez-Márquez & Peretti 2010; Huber 1995; Kaster & Jakob 1997). In contrast, only some vibrations appear to originate from the female during mating, as sometimes females tapped briefly with their anterior legs

(Huber & Eberhard 1997). Thus the negative correlation between immunocompetence and reproduction may not be related to behavior in females.

The immunocompetenee cost of mating in females may come from a down-regulation to save energetic resources for the future expenditures of oviposition. For example, it may be that females need to find a particular place for oviposition or use specific resources for egg provisioning that may be energetically demanding. Furthermore, as has been demonstrated in other invertebrates, males could transfer seminal produets during copulation, which may induce female diversion of resources to egg produetion at the expense of female immunity (reviewed in Lawniczak et al. 2006).

In relation to the cost of oviposition, females may face resource allocation dilemmas during this period so that immune ability is again compromised. Reductions in LA levels, however, may be alternatively explained by mechanisms apart from energetic demands (e.g., Siva-Jothy et al. 1998). In some insects, juvenile hormone down-regulates immune ability during mating and oviposition (Rolff & Siva-Jothy 2002), although there are important exceptions to this (e.g., Córdoba-Aguilar et al. 2011).

Agonistic interactions also led to reduced immunocompetence, as found in other arthropods (e.g., Contreras-Garduño et al. 2006, 2009). In pholcids, agonistic interactions are common and could elicit costs in at least three different situations (Jakob 1999). One first cost is due to reduced access to food. For example, Jakob (1991) found in *Holocnemus pluchei* (Scopoldi 1763) that gregarious individuals fed less than solitary individuals. A second context is related to the cost of being injured or cannibalized in aggressive interactions with conspecifics. The final context is the cost of web site and/ or web investment. Such a cost will emerge if, for example, a spider is driven away from its own web and needs to find another web or build a new one (Jakob 1999). As a complement to the immunocompetence costs measured here, future studies should seek to quantify the energetic resources spent during aggression. One likely variable appropriate for such an investigation is muscular lipid-based fat, as in other insects (Contreras-Garduño et al. 2006).

Despite our support for the negative effect of reproductive activities on immunocompetence, other studies have found contrary results (e.g., Shoemaker et al. 2006; Valtonen et al. 2010; Córdoba-Aguilar et al. 2011). There are different explanations for such disparate findings. One is that the difference may be due to the particular biology of some species. For example, Valtonen et al. (2010) documented that mating enhanced resistance against fungal infections in the mealworm beetle, *Tenebrio molitor*, a species whose males and females are highly promiscuous (e.g., Eady 1995). A second explanation is that the condition of the animal may play a role at the time reproductive aetivities take place. It is known that resource allocation conflicts, when immunity is involved, will be more dramatic for animals in poor condition than for animals in good condition (Sheldon & Verhulst 1996). Related to this, studies carried out under field and laboratory conditions may have used animals that differed widely in condition. Even in the laboratory, differences in condition may be found. If food is provided, animals may eat more to compensate for the energetic demands imposed by immunological costs (e.g., Povey et al. 2009), a situation that is usually not controlled. One final explanation is that the immunological cost exists, but that finding it depends on the immune parameter being used. This is because it is known that not all immune parameters may indicate animal condition or resistance (e.g., Adamo 2004). In fact, a number of arthropod studies have used phenoloxidase activity, a key effector during immune response (reviewed by González-Santoyo & Córdoba-Aguilar 2012), for detecting immunological costs of different energetically demanding activities that include reproductive activities (reviewed by Lawniczak et al. 2006). Paradoxically, a recent review concluded that phenoloxidase activity does not indicate the host's resistance and only under some cireumstances correlates with the host's condition (reviewed by González-Santoyo & Córdoba-Aguilar 2012). Given the above potential sources of noise, we concur with Valtonen et al. (2010) that it is still premature to conelude that reproductive activities impair immunocompetence in arthropods.

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